The American Journal of Pathology, Vol. 179, No. 4, October 2011

DOI: 10.1016/j.ajpath.2011.06.023

Immunopathology and Infectious Diseases

A Detrimental Role for Invariant Natural Killer T Cells in the Pathogenesis of Experimental Dengue Virus Infection

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Dengue virus (DENV), a member of the mosquito-borne flaviviruses, is a serious public health problem in many tropical countries. We assessed the *in vivo* physiologic contribution of invariant natural killer T (iNKT) cells, a population of nonconventional lipid-reactive $\alpha\beta$ T lymphocytes, to the host response during experimental DENV infection. We used a mouse-adapted DENV serotype 2 strain that causes a disease that resembles severe dengue in humans. On DENV challenge, splenic and hepatic iNKT cells became activated insofar as CD69 and Fas ligand up-regulation and interferon-γ production. C57BL/6 mice deficient in iNKT cells ($J\alpha18^{-/-}$) were more resistant to lethal infection than were wild-type animals, and the phenotype was reversed by adoptive transfer of iNKT cells to $J\alpha18^{-/-}$ animals. The absence of iNKT cells in $J\alpha18^{-/-}$ mice was associated with decreased systemic and local inflammatory responses, less liver injury, diminished vascular leak syndrome, and reduced activation of natural killer cells and neutrophils. iNKT cell functions were not necessary for control of primary DENV infection, after either natural endogenous activation or exogenous activation with the canonical iNKT cell agonist α-galactosylceramide. Together, these data reveal a novel and critical role for iNKT cells in the pathogenesis of severe experimental dengue disease. ***(Am J Pathol 2011, 179:1872–1883; DOI: 10.1016/j.ajpath.2011.06.023)***

Dengue virus (DENV), a flavivirus of the *Flaviviridae* family, is a serious public health problem in tropical and subtropical areas. In the last 60 years, the incidence, distribution, and clinical severity of dengue-related diseases have increased dramatically.[1,2] There are an estimated 50 million to 100 million DENV infections each year, of which approximately 500,000 are severe dengue hemorrhagic fever, and 24,000 result in death.[2,3] DENV is a single-stranded RNA virus that is transmitted to humans by *Aedes* mosquitoes, primarily *Aedes aegypti*. Infection with any of the four serotypes of DENV results in clinical symptoms that range from classic dengue fever to severe dengue hemorrhagic fever and shock syndrome, as defined by the World Health Organization.[4] Severe forms are life-threatening and are characterized by hemorrhagic manifestations, hemoconcentration, thrombocytopenia, and increased vascular permeability.[1,5–10] Despite growing public health concerns, currently there is no vaccine and no specific thera-

Supported in part by the Institut National de la Santé et de la Recherche Médicale, the CNRS, the University of Lille Nord de France, the Pasteur Institute of Lille, the French National Research Agency (ANR) under references ANR-08-MIEN-021-01 (FT) and ANR-07-MIME-103-02 (BR), Fondation pour la Recherche Médicale (FRM allergy DAL 2007 0822007), Fond Européen de Développement Régional (FEDER Asthme 1575-32168), and the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq, Brazil). J.R. was supported by a postdoctoral fellowship from the ANR. R.G. and R.E.M. were recipients of a doctoral fellowship from the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq, Brazil). S.I. and C.P. were recipients of a doctoral fellowship from the Ministère de l'Education Nationale de la Recherche et Technique. V.Q., B.R., and F.T. are supported by the CNRS, and C.F. by INSERM.

Accepted for publication June 9, 2011.

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peutic agents. Treatment is supportive and may require administration of intravenous fluids, which places a serious burden on health systems in low- income countries.

The pathogenesis of DENV remains poorly understood and involves a complex interplay of viral and host factors. Risk factors for severe disease include age, viral serotype and genotype, host genetic background, and previous infection with a distinct serotype.[1,11,12] These factors seem to interact to enhance viral replication and viral load. Ultimately, DENV can interact with target cells including dendritic cells,[13] monocytes and macrophages,[14] hepatocytes, and endothelial cells,[15,16] resulting in production of immune and inflammatory mediators that shape innate and acquired immune responses and the risk of disease. It has been suggested that dysfunction of vascular endothelial cells leading to plasma leakage is mediated by host immune response.[9] Production of high levels of proinflammatory cytokines including tumor necrosis factor-α, IL-1β, IL-6, and interferon-γ (IFN-γ) has been reported in patients with severe dengue disease.[7,10] However, it is not clearly understood how this massive cytokine production is induced and eventually controlled. Similarly, the nature of effector and bystander cells able to control DENV challenge during the early phase of infection remains elusive.

Natural killer T (NKT) cells are a heterogeneous population of innate/memory nonconventional $\alpha\beta$ T lymphocytes that recognize, through their T-cell receptors (TCRs), self and foreign (glyco)lipid antigen presented by the nonpolymorphic major histocompatibility complex class I–like protein CD1d (reviewed in Refs. 17 to 20). CD1d-restricted NKT cells are divided into two subsets: invariant (iNKT cells, or type I NKT cells), which represent the predominant subset and express exclusively an invariant TCR-α chain (Vα14Jα18 in mice), and variant (vNKT cells, or non-invariant or type II NKT cells), which express more diverse TCRs.[17,19,20] An important feature of NKT cells is that after endogenous or exogenous TCR-mediated activation, they quickly produce a wide array of cytokines. Through this unique property, NKT cells perform potent immunoregulatory functions in autoimmune and inflammatory diseases, cancer, and infection.[19–21] For example, exposure of iNKT cells with the high-affinity lipid α-galactosylceramide (α-GalCer) promptly induces the production of large amounts of T helper (Th) 1– (IFN-γ), Th2- (IL-4), and Th17- (IL-17A/F) associated cytokines that influence the outcome of developing or ongoing immune reactions with, in general, beneficial effects on cancer and infection.[22,23] Although the natural function of vNKT cells in anti-viral immunity and in control of viral replication and disease is only vaguely understood, the physiologic role of iNKT cells in viral infection has been extensively studied using mice lacking the TCR *Jα18* segment (*Jα18*$^{-/-}$ mice). These mice are exclusively devoid of iNKT cells; however, the other lymphoid cell lineages are intact. After comparison of the phenotypes of *Jα18*$^{-/-}$ and wild-type (WT) animals, it was reported that the role of iNKT cells during experimental viral infection can vary according to the virus and experimental conditions.[20,24–26] For example, during infection with influenza A virus, herpes simplex virus types 1 and 2, and lymphocytic choriomeningitis virus, iNKT cells have a positive role in anti-viral immune responses and virus-associated disease,[27–32] whereas during infection with Sendai virus and herpes simplex virus type 2 (only in aged mice), iNKT cells are rather deleterious.[33,34] However, iNKT cells do not seem to participate in anti-viral immunity or control of viral replication during infection with encephalomyocarditis virus and murine cytomegalovirus.[35–37] The potential role of iNKT cells in experimental viral infections has also been studied using CD1d-deficient mice, which lack both iNKT cells and vNKT cells. Although it is not possible to differentiate between a role of iNKT cells, vNKT cells, or the CD1d molecule, comparison of CD1d-competent and CD1d-deficient mice has suggested that NKT cells might positively contribute to the immune response to infection with respiratory syncytial virus, Theiler's murine encephalomyelitis virus, encephalomyocarditis virus, and murine cytomegalovirus.[35,37–39] Although there are relatively few iNKT cells in the human system, there is evidence for an antiviral role of these cells.[40–43] Of note, the potential role of NKT cells during flavivirus infection has not yet been addressed. The objective of the present study was to investigate the natural role of iNKT cells during experimental DENV infection by comparing the disease outcome in WT and iNKT cell–deficient mice. The findings suggest a crucial role of iNKT cells in driving the systemic and local inflammatory responses that cause the disease associated with DENV infection. In addition, it is shown that endogenous or exogenous activation of iNKT cells does not lead to control of DENV replication.

Materials and Methods

Mice

Eight- to 10-week-old female WT C57BL/6 (H-2D^b) mice were purchased from Centre d'Elevage Janvier (Le Genest-St. Isle, France) and maintained using autoclaved food and water available *ad libitum* under specific pathogen–free conditions. *Jα18*$^{-/-}$ mice, backcrossed at least 10 times in C57BL/6, were a gift from Dr. Masaru Taniguchi (RIKEN Yokohama Institute, Yokohama, Japan).[22] For infectious protocol, the mice were kept in isolated ventilated cages in the biohazard animal unit of the Transgenose Institute (CNRS, Orléans, France). All experimental procedures were approved by and complied with the ethical and animal experiment regulations of the French government.

Virus

The mouse-adapted DENV serotype 2 strain P23085 was obtained from the State Collection of Viruses (Moscow, Russia) and adapted as previously described.[44] The nucleic acid sequence of a portion for E and NS1 genes of DENV strain P23085 has been deposited previously at GenBank under accession No. AY927231.1. The partial sequence shows 98% identity with the corresponding region of a human DENV serotype 2 isolate. For the present set of experiments, the last two passages of DENV strain P23085 was performed in LLC-MK2 cells (kidney, Rhesus monkey; American Type Culture Collection, Manassas, VA) to produce stocks, which were stored

in DMEM (Dulbecco's modified Eagle's medium; Sigma-Aldrich Corp., St. Louis, MO) at −80°C. To calculate virus titer, expressed as LD_{50}, groups of 10 mice were inoculated i.p. with serial dilutions of the virus, and lethality was recorded as described previously.[45–47] The titer of DENV stock was 10^5 LD_{50}/mL, or 2×10^6 plaque-forming units (PFUs)/mL based on plaque assay on LLC-MK2 cells.

Infection and Assessment of Disease

The virus-containing LLC-MK2 supernatants were diluted in endotoxin-free PBS and injected i.p. into mice. Mock-treated animals were inoculated with DMEM supernatants from noninfected LLC-MK2 cells similarly diluted. Mice were inoculated i.p. with 30 LD_{50} DENV, a lethal dose that caused death of all animals between days 6 and 8. One LD_{50} corresponds to the inoculum necessary to kill 50% of 4-week-old BALB/c mice, a more susceptible strain,[48] and corresponds to approximately 20 PFUs. Clinical manifestations, in particular hypernociception, were observed at about day 3. Within 5 or 6 days after infection, mice demonstrate other symptoms including ruffling of the fur, slowing of activity, and weight loss. Worsening of clinical and laboratory factors ensued, with death from day 7, depending on the inoculum used. Disease was assessed by measuring vascular leakage, thrombocytopenia, plasma concentrations of cytokines and chemokines, liver damage, body weight loss, viral load, and lethality. At day 5, blood was recovered for serum preparation and hematologic analysis. Spleen and liver samples were recovered for cytokine measurement and viral titration. Liver samples were also used for histologic analysis (day 6). At days 3 and 5, spleen and liver were used for analysis via fluorescence-activated cell sorting.

Hematologic Parameters and Histopathologic Analysis

Blood was obtained from the brachial plexus in heparin-containing syringes at indicated times. Platelets and relative percentages of granulocytes were counted using a Coulter counter (S-Plus Jr; Beckman Coulter France SAS, Roissy, France), and hematocrit in a hematocrit centrifuge. Hematocrit concentration and extravasation of Evans blue dye into the tissues was used as an index of increased vascular permeability, as previously described.[45,48] A portion of liver was obtained from mice euthanized at day 6 and was immediately fixed in 4% buffered formalin, and tissue fragments were embedded in paraffin. Tissue sections 4 μm thick were stained using H&E and examined under light microscopy for inflammatory changes using a semiquantitative score.

Titration of Virus

Mice were assayed for viral titers in spleen and liver at 4 and 6 days after infection. For virus recovery, the organs were collected aseptically and stored at −70°C until assayed for DENV. Tissue samples were weighed, ground using a mortar and pestle, and prepared as 10% (w/v) homogenates in DMEM without fetal bovine serum. Viral load in supernatant of tissue homogenates was assessed using direct plaque assays of LLC-MK2 cells overlayed on carboxymethylcellulose (Sigma-Aldrich Corp.). In brief, samples of organ homogenates were diluted serially, and 0.5-mL was placed in duplicate into each of six wells of LLC-MK2 cell monolayers and incubated for 1 hour. An overlay solution containing 199 medium (Gibco-BRL, Invitrogen Corp.) with Earle's salts, L-glutamine, and 3% fetal bovine serum in 1.5% carboxymethylcellulose was added to each well, and the cultures were incubated for 9 days. Cultures were stained with crystal violet for enumeration of viral plaques. The results were measured as PFUs/100 mg tissue weight. The limit of detection of the assay was 100 PFUs/100 mg tissue.

Quantification of Cytokine and Chemokine Concentration

Serum samples and tissue extracts were analyzed for IFN-γ (eBiosciences, CliniSciences SA, Montrouge, France) and IL-6, tumor necrosis factor-α, IL-1β, CXCL1/KC, and IL-12 p40 (R&D Systems Europe, Ltd., Abingdon, Oxfordshire, England) using enzyme-linked immunosorbent assay kits according to manufacturer instructions.

Preparation of Liver and Spleen Cells

Livers were perfused via the venous sinus with PBS to remove circulating blood cells. Perfused livers were finely minced and treated via enzymatic digestion for 20 minutes at 37°C in RPMI medium containing 1 mg/mL collagenase type VIII and 1 μg/mL DNase type I (both from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). After washing, cell suspensions were resuspended in a 36% Percoll gradient, carefully layered onto 72% Percoll, and centrifuged for 30 minutes at 2300 rpm, without brake, at 22°C. Hepatic leukocyte populations collected at the interface were washed in PBS/2% fetal calf serum. Spleens were collected and homogenized using 100 μmol/L cell strainers (BD Biosciences, Franklin Lakes, NJ). Red blood cells were removed using lysis buffer (Sigma-Aldrich Corp.).

Flow Cytometry

Monoclonal antibodies (mAbs) against mouse TCRβ [fluorescein isothiocyanate (FITC)–conjugated], NK1.1 [phycoerythrin (PE)– or peridinin chlorophyll protein–cyanine 5.5 (PerCp-Cy5.5)–conjugated], CD5 [FITC- or allophycocyanin (APC)–conjugated], CD4 (FITC-conjugated), CD8 (APC-conjugated), CD69 (PE-, PerCp-Cy5.5–, or APC-conjugated), CD178 [Fas-ligand (Fas-L)] (biotinylated), streptavidin (PE-Cy7–conjugated), IFN-γ (Alexa Fluor 647–conjugated), IL-4 (APC-conjugated), CD11c (PE-Cy7–conjugated), CD11b (PerCp-Cy5.5–conjugated), Ly6G (Alexa Fluor 647–conjugated), CD62-L (PE-conjugated), and isotype controls were all purchased from BD Pharmingen (BD Biosciences). MAbs against granzyme B (FITC-conjugated), CD107α (PE-conjugated), and isotype controls were purchased from eBiosciences. PE-con-

jugated PBS-57 glycolipid-loaded CD1d tetramer was obtained from the National Institute of Allergy and Infectious Diseases Tetramer Facility at Emory University (Atlanta, GA). To analyze iNKT cells, mononuclear cell suspensions were incubated with appropriate dilutions of PBS-57–loaded CD1d tetramer–PE for 30 minutes in PBS containing 2% fetal calf serum and 0.01% NaN_3. Cells were then washed and stained for other cell surface markers. For intracellular staining, cells were fixed in 1% paraformaldehyde in PBS for 10 minutes, resuspended in PBS containing 2% fetal calf serum and 0.1% saponin (permeabilization buffer), and incubated using Alexa Fluor 647–conjugated mAb against IFN-γ, IL-4, or control rat IgG1 mAb or with granzyme B-FITC or control rat IgG2b mAb. Cells were acquired on a cytometer (BD FACSCanto II; BD Biosciences) and analyzed using FlowJo software (TreeStar, Inc., Ashland, OR).

NKT Purification and Adoptive Transfer

For adoptive transfer experiments and to prevent activation of iNKT cells, NKT cells were purified from the livers of naïve animals using CD5 and NK1.1 Abs. In brief, liver cells from WT donor mice were stained using anti-NK1.1 (PE-conjugated) and anti-CD5 (APC-conjugated) mAbs. Labeled cells were isolated using a FACSAria cell sorter and BD FACSDiva software (both from BD Biosciences). NKT ($CD5^+$ $NK1.1^+$) cell purity after sorting was consistently greater than 98%. About 85% to 90% of sorted hepatic NKT cells also stained positive using PBS-57–loaded CD1d tetramer. In parallel, iNKT cells were sorted on the basis of PBS57-loaded CD1d tetramer and TCRβ staining (>98% pure). *Jα18*$^{-/-}$ recipient mice were inoculated i.v. with either 1×10^6 $CD5^+$ $NK1.1^+$ or PBS57-loaded CD1d tetramer$^+$ TCRβ^+ cells or with the same volume of medium alone at 18 hours before DENV infection.

Inoculation of α-GalCer

Mice were inoculated i.v. with α-GalCer (Axxora Life Sciences, Coger SA, Paris, France), 2 μg per mouse, 1 hour before infection. The α-GalCer used in the present study prophylactically protected mice against lethal challenges with influenza A virus (data not shown) or *Streptococcus pneumoniae* [Ivanov et al. (submitted for publication)].

Statistical Analysis

Data were analyzed using commercially available software (PRISM version 4; GraphPad Software, Inc., San Diego, CA). Results are given as mean ± SEM. Statistical significance of difference between experimental groups was calculated using one-way analysis of variance with a Bonferroni posttest or a two-tailed unpaired Student's *t*-test. The possibility of using these parametric tests was assessed by determining whether the population was gaussian and the variance was equal (Bartlett's test). Survival of mice was compared using Kaplan-Meier analysis and the log-rank test. $P < 0.05$ was considered significant; n = number of mice.

Results

iNKT Cells Become Activated During DENV Infection

An important feature of iNKT cells during stressful conditions is rapid activation to initiate innate and adaptive immune responses. We first investigated the activation status of iNKT cells during the course of experimental DENV infection. As shown in Figure 1A, and relative to mock-treated mice, the frequency and absolute number of splenic iNKT cells remained stable at 3 days after infection but decreased significantly by approximately 50% at day 5 after infection. This apparent disappearance of iNKT cells can occasionally occur during infection, a phenomenon due to TCR internalization[49–51] or to activation-induced cell death.[52,53] To further study the activation status of iNKT cells, we monitored the expression of the early activation marker CD69. As shown in Figure 1B, the level of CD69 expression on iNKT cells was significantly increased at day 5 after infection, whereas it was unchanged at day 3 after infection. Fas-L is up-regulated on activated iNKT cells and is important in concanavalin A–induced hepatocyte damage and antigen-specific cytotoxicity.[54,55] Compared with controls, iNKT cells express enhanced levels of Fas-L at 5 days, but not at 3 days, after DENV challenge (Figure 1B). Of note, NK ($CD5^-$ $NK1.1^+$) cells also increased CD69 and FAS-L expression with the same kinetic.

During infection, iNKT cells are among the first to produce cytokines, which have multiple effects on other immune and nonimmune cells. Therefore, we determined whether iNKT cells expressed cytokines during DENV infection. Compared with mock treatment, DENV infection resulted in an increased frequency of splenic iNKT cells expressing IFN-γ at 5 days, but not at 3 days, after infection (Figure 1C). In contrast, iNKT cells remained negative for IL-4 and IL-17 (data not shown). Of note, iNKT cell activation was also observed in the liver of DENV-infected mice at 5 days after infection (data not shown). Together, these results demonstrate that DENV infection is accompanied by systemic activation of iNKT cells.

Mice Deficient in iNKT Cells Survive to Lethal DENV Challenge

Mice infected with the mouse-adapted DENV serotype 2 strain P23085 manifest disease that shares several features with human clinical disease.[44–47] In this system, disease is associated with virus replication, acute systemic inflammation, thrombocytopenia, and increased vascular permeability, which eventually lead to shock and death. To address the potential role of iNKT cells in the development and control of the immunopathologic condition, WT and iNKT cell–deficient mice (*Jα18*$^{-/-}$) were infected with a lethal dose of DENV serotype 2 strain P23085 and monitored daily for clinical signs including change in body weight and survival. Infection of WT mice was associated with rapid weight loss starting at day 5 after infection (Figure 2A), and all mice died of DENV

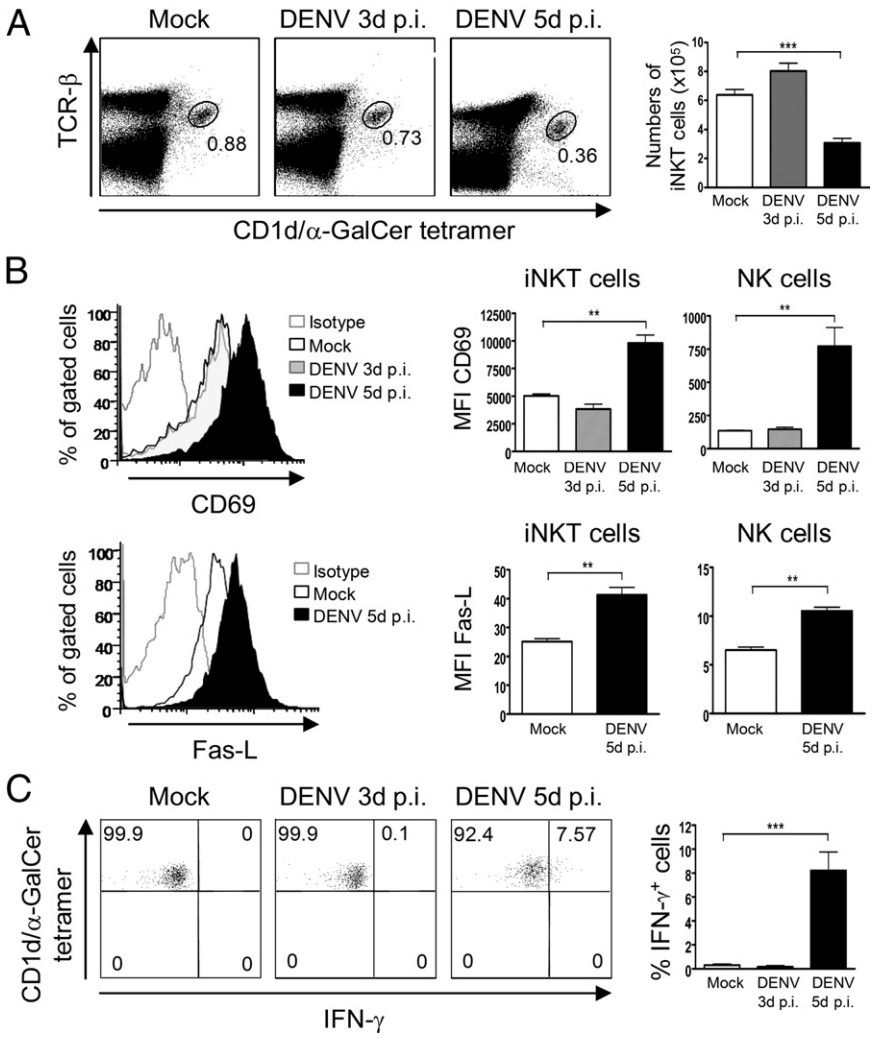

Figure 1. Activation of iNKT cells during the course of DENV infection. Mice were inoculated with a lethal dose (30 LD_{50}) of the mouse-adapted DENV serotype 2 strain P23085, and splenic iNKT cells were analyzed at 3 and 5 days after infection (p.i.) **A:** Percentages of gated CD1d/α-GalCer tetramer$^+$ TCRβ^+ (iNKT) cells in mock-treated or DENV-infected mice at 3 and 5 days after infection. Representative dot plots are shown. **Right panel:** Absolute number of splenic iNKT cells. Data are given as mean ± SEM of three independent experiments (n = 6 to 10). No major changes in total numbers of splenic cells were noted between mock-treated and DENV-infected mice (data not shown). **B:** Expression of CD69 and Fas-L on the surface of iNKT cells and CD5$^-$ NK1.1$^+$ (NK) cells. Histogram overlay represents the expression of CD69 and Fas-L on gated iNKT cells from either mock-treated or DENV-infected mice at 3 or 5 days after infection. Bar histograms represent the mean expression (MFI) of CD69 and Fas-L on iNKT and NK cells. Data are given as mean MFI ± SEM (n = 6). **C:** Analysis of *ex vivo* cytokine production by iNKT cells at 3 and 5 days after infection. Gated iNKT cells were analyzed for intracellular IFN-γ production. Gates were set based on the isotype control. Representative dot plots are shown. Mean percentages ± SEM of splenic iNKT cells positive for IFN-γ are represented on the bar histograms (n = 3 to 10). Significant differences are designated using one-way analysis of variance with a Bonferroni posttest (**A–C**) or a two-tailed unpaired Student's t-test (**B**, **lower panel**). $^{**}P < 0.01$, $^{***}P < 0.001$.

infection at 8 days after infection (Figure 2B). *Jα18*$^{-/-}$ mice developed fewer clinical manifestations including ruffling of fur, slowing of activity, and weight loss, and 70% of animals survived to infection at day 21 (Figure 2). To further demonstrate the involvement of iNKT cells in DENV-associated death, *Jα18*$^{-/-}$ mice were reconstituted with iNKT cells from naïve animals just before DENV lethal challenge. To this end, liver iNKT cells were purified on the basis of CD5 and NK1.1 expression, a procedure that leads to an incompletely pure iNKT cell population (85% to 90% purity) but has the advantage of leaving iNKT cells in a nonactivated state. The adoptive transfer of NKT cells did not significantly restore the phenotype to that of WT animals because only 40% of reconstituted *Jα18*$^{-/-}$ mice died of DENV infection between days 6 and 10 after infection (Figure 2). This effect may be due to the presence of vNKT cells in the cellular preparation (approximately 10%; data not shown). To verify this, iNKT cells were sorted on the basis of PBS-57–loaded CD1d tetramer and TCR-β staining, a procedure that leads to approximately 98% of pure, albeit slightly activated, iNKT cells. Of mice reconstituted with PBS-57–loaded CD1d tetramer$^+$ TCR-β$^+$, 80% died of infection (Figure 2). Thus, the data highlight the importance of iNKT cells in DENV-associated lethality.

iNKT Cell–Deficient Mice Develop Less Severe Disease after DENV Infection

DENV-associated lethality is preceded by significant changes in hematocrit, vascular permeability, platelet counts, and cytokine concentrations.[1,5,6,8–10,56] Inasmuch as vascular leak syndrome is a hallmark of severe DENV infection in humans,[6,8] we measured hematocrit and Evans blue permeability in DENV-infected WT and *Jα18*$^{-/-}$ mice. Compared with mock-treated mice, the hematocrit concentration was higher at day 5 after infection in DENV-infected WT animals (Figure 3A). The virus-associated hemoconcentration in WT mice was not observed in *Jα18*$^{-/-}$ mice. Evans blue leak assays demonstrated severe vascular leakage in livers of DENV-infected WT animals (Figure 3A). In contrast, vascular leakage was of lower amplitude in the liver of infected *Jα18*$^{-/-}$ mice. Thrombocytopenia occurs in DENV-infected patients. As seen in Figure 3B, there was a significant decrease in platelet counts in infected WT and *Jα18*$^{-/-}$ mice, albeit the decrease was less intense in the *Jα18*$^{-/-}$ mice. Neutrophils may become activated, accumulate in tissues, and contribute to organ damage in the

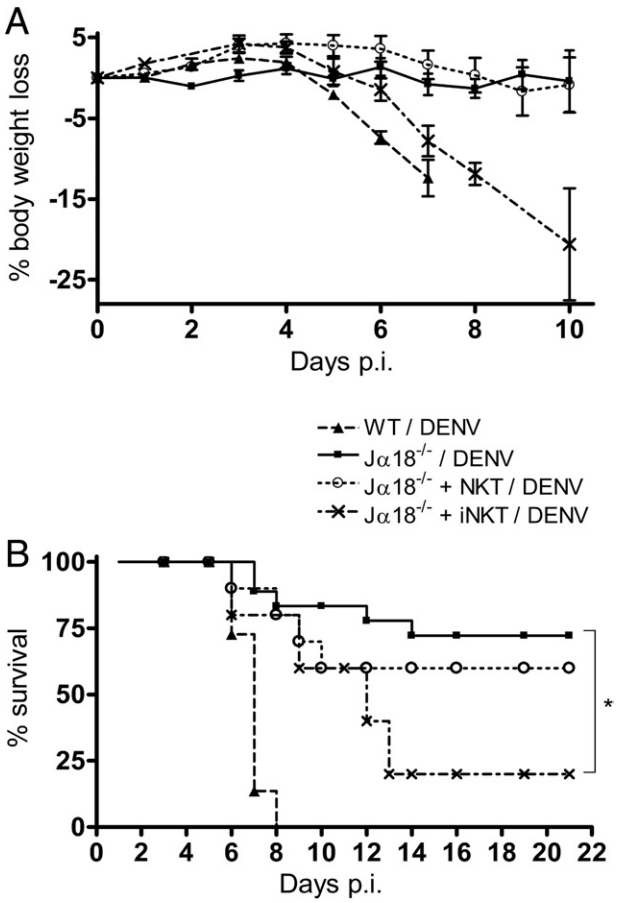

Figure 2. Variation of body weight and survival rates in WT and $J\alpha18^{-/-}$ mice reconstituted or not with iNKT cells. Age-matched WT or $J\alpha18^{-/-}$ mice, either reconstituted or not with iNKT cells, were infected with a lethal dose (30 LD_{50}) of DENV and then weighed daily and observed until death. $J\alpha18^{-/-}$ mice were injected i.v. with either 1×10^6 cell-sorted $CD5^+$ $NK1.1^+$ (NKT) cells or PBS-57–loaded CD1d $tetramer^+$ TCR-β^+ (iNKT) cells or PBS at 18 hours before DENV challenge. **A:** Body weight loss is expressed as a percentage of the animal's initial weight. **B:** Percentages of survival (n = 10 to 22, at least two independent experiments). Log-rank test for comparisons of Kaplan-Meier survival curves indicated a significant increase in the mortality of $J\alpha18^{-/-}$ mice reconstituted with iNKT cells compared with non-reconstituted $J\alpha18^{-/-}$ animals. p.i., after infection. *P = 0.024.

context of DENV infection.[57] Compared with mock-treated WT mice, in DENV-infected WT mice, the percentage of circulating neutrophils was augmented significantly. This increase in neutrophils was not observed in the blood of infected $J\alpha18^{-/-}$ mice. Of note, the frequency of monocytes and lymphocytes was not dramatically different between infected WT and $J\alpha18^{-/-}$ mice (data not shown). We next measured the concentrations of inflammatory cytokines in the serum of infected mice. The concentrations of IL-6 and IFN-γ were strongly increased in plasma of infected WT animals (Figure 3C). Concentrations of these cytokines were significantly lower in infected $J\alpha18^{-/-}$ mice. To compare the extent of tissue injury, livers from WT and $J\alpha18^{-/-}$ mice were analyzed at day 6 after infection. In infected WT mice, H&E-stained liver sections clearly revealed signs of congestion, hemorrhage, hepatocyte degeneration, and necrosis (Figure 3D). In marked contrast, the architecture of the liver was preserved in infected $J\alpha18^{-/-}$ mice. In aggregate, the data demonstrate that the absence of iNKT cells in $J\alpha18^{-/-}$ mice prevented DENV-associated vascular leakage and tissue damage.

Mice Deficient in iNKT Cells Display Reduced Production of Inflammatory Cytokines

The cytokine "storm" that follows infection correlates with the severity of dengue disease and is believed to contribute to the pathogenesis of severe dengue.[8,10,56]

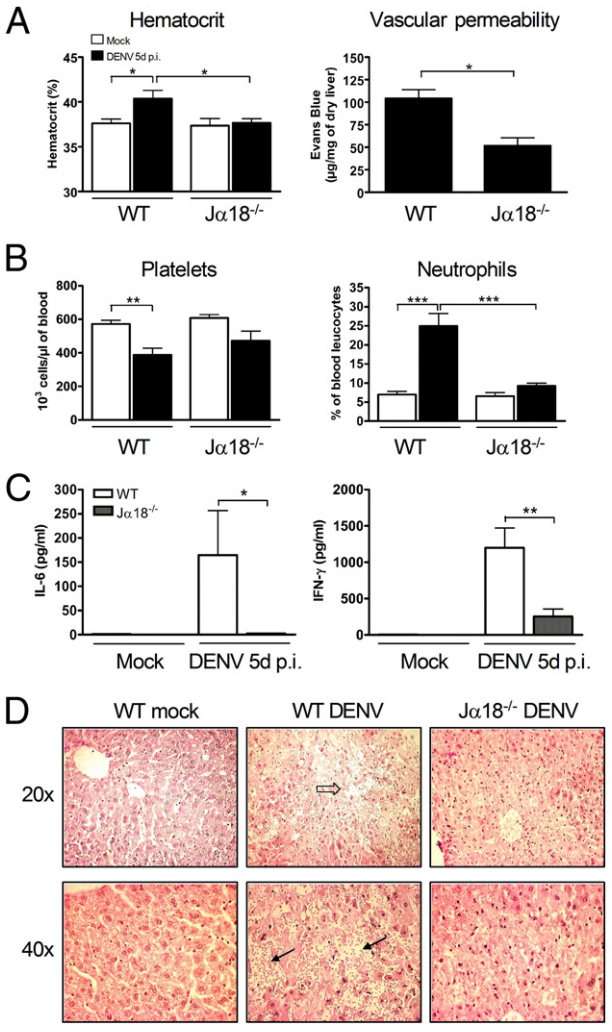

Figure 3. Analysis of disease in WT and $J\alpha18^{-/-}$ mice infected with a lethal dose of DENV. Blood from mock-treated or DENV-infected WT and $J\alpha18^{-/-}$ mice was collected at 5 days before infection (p.i.) and analyzed for hematologic parameters. **A:** Hematocrit in blood is shown as percent volume occupied by red blood cells (**left panel**). Evans blue extravasation in liver was used as an index of increased vascular permeability. Changes in vascular permeability are shown as micrograms Evans blue per milligram dry tissue (**right panel**). **B:** The number of platelets is shown as number $\times 10^3/\mu L$ blood, and neutrophils as percentages. **C:** Serum from mock-treated or DENV-infected mice was collected at day 5 after infection, and concentrations of IL-6 and IFN-γ were measured using an enzyme-linked immunosorbent assay. **A–C:** Data are given as mean ± SEM (n = 6 to 11 mice per group; at least two independent experiments). Significant differences were designated using one-way analysis of variance with a Bonferroni posttest (**A–C**) or a two-tailed unpaired Student's t-test (**A**, **right panel**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **D:** Representative H&E-stained liver sections of mock-treated and DENV-infected mice at 6 days after inoculation. **Upper panel: Arrow** indicates necrosis. **Lower panel: Arrows** indicate hemorrhage.

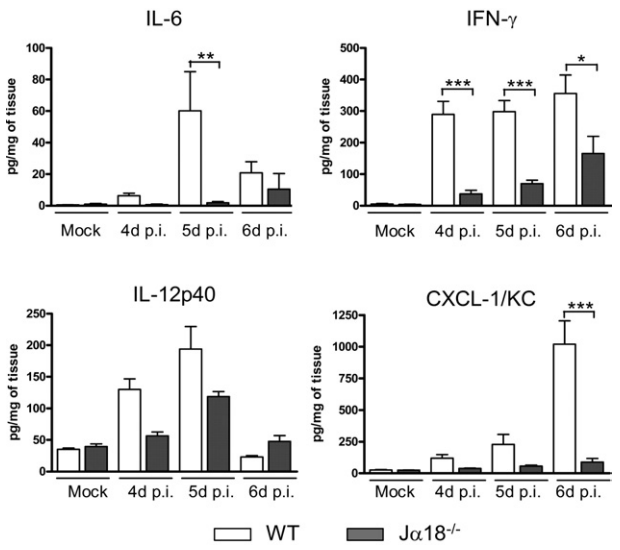

Figure 4. Analysis of the inflammatory cytokine response in WT and *Jα18*$^{-/-}$ mice infected with a lethal dose of DENV. Spleens from DENV-infected WT and *Jα18*$^{-/-}$ mice were collected at days 4, 5, and 6 after infection (p.i.). Tissues were homogenized, and the concentrations of IL-6, IFN-γ, IL-12 p40, and CXCL-1/KC were analyzed using an enzyme-linked immunosorbent assay. Values represent mean ± SEM (n = 4 to 8, two independent experiments). Significant differences between groups are designated by using one-way analysis of variance with a Bonferroni posttest. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

As shown in Figure 4, increased concentrations of IL-6, IFN-γ, and IL-12p40 were observed in the spleen of WT animals at days 4 to 6 after infection, at both protein and transcript (data not shown) levels. Similarly, DENV-infected WT mice produced a higher concentration of the inflammatory chemokine CXCL-1/KC, which is known to rapidly mobilize and activate neutrophils, from day 4 after infection, with a peak at day 6. The magnitude of these inflammatory markers was significantly reduced in the spleens of infected *Jα18*$^{-/-}$ mice. Altogether, these results demonstrate that iNKT cells drive the systemic inflammatory response that accompanies DENV infection.

Jα18$^{-/-}$ *Mice Exhibit Decreased Activation of NK Cells and Neutrophils During DENV Infection*

In vivo activation of iNKT cells triggers downstream stimulation, and sometimes expansion, of various immune cells including NK cells, neutrophils, and conventional T lymphocytes, which are important during DENV infection.[57–59] Thus, we measured the effect of endogenous iNKT cell activation on the frequency and activation of these other cells. DENV infection was associated with reduced frequency of NK cells (Figure 5A) and enhanced percentage of neutrophils (Figure 5B) in the spleen of WT mice. In contrast, the frequency of both NK cells and neutrophils was not modulated in infected *Jα18*$^{-/-}$ mice. We next measured the level of surface markers associated with cell activation on these cell types. NK cells from infected WT mice expressed higher levels of CD69 (Figure 5A), whereas this induction was significantly lower in NK cells from infected *Jα18*$^{-/-}$ mice. Moreover, expression of granzyme B (Figure 5) and CD107α (data not shown), two molecules known to participate in cellular cytotoxicity, was strongly up-regulated in or on NK cells from WT mice. This induction was less intense in iNKT cell–deficient animals. Activated iNKT cells can *trans*-stimulate NK cells to produce IFN-γ. As seen in Figure 5A, splenic NK cells from WT-infected mice labeled positively for IFN-γ at day 5 after infection, whereas the frequency of IFN-γ–positive NK cells was much lower in iNKT cell–deficient animals. Neutrophils become activated during DENV infection.[57] Neutrophils expressed

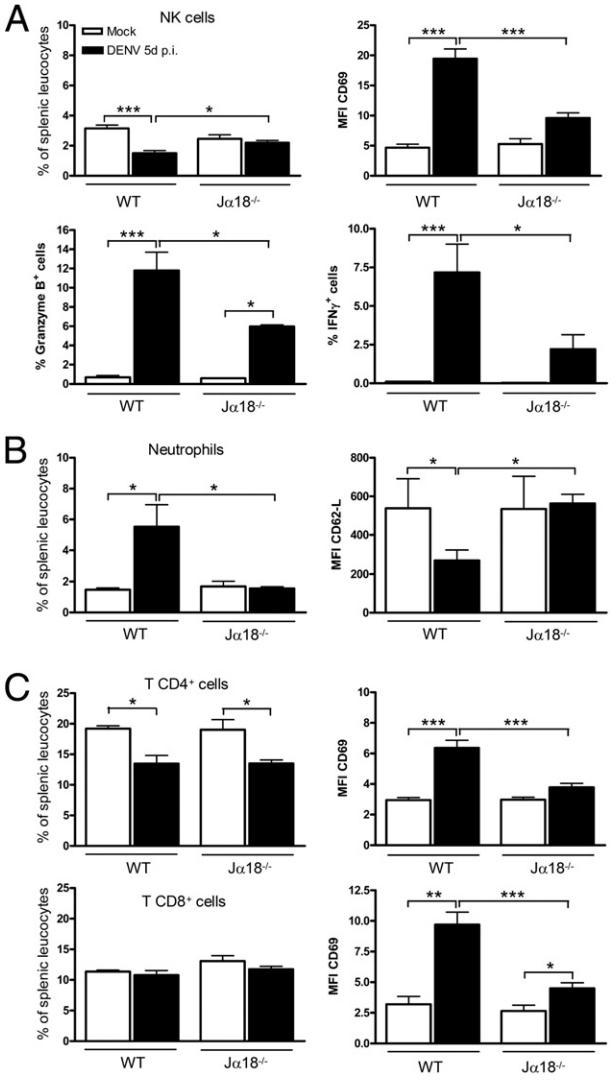

Figure 5. Analysis of NK cell, neutrophil, and T-lymphocyte frequency and activation in infected WT and *Jα18*$^{-/-}$ mice. At 5 days after infection (p.i.), splenocytes from infected mice were recovered and analyzed for NK cell, neutrophil, and T-cell frequency and activation status. **A:** Mean ± SEM percentage of gated CD5^{-} NK1.1^{+} cells from mock-treated and infected WT or *Jα18*$^{-/-}$ mice (**upper left panel**). NK cells were also analyzed for cell surface CD69 expression (mean MFI ± SEM) (**upper right panel**) and intracellular granzyme B and IFN-γ expression (mean percent positive cells ± SEM) (**lower panels**). **B:** Frequency ± SEM of CD11c^{+} Ly6G^{+} cells (neutrophils) and expression of CD62-L (mean MFI ± SEM) on gated neutrophils. **C:** Mean ± SEM percentages of CD4^{+} and CD8^{+} T cells (**upper panels**). Expression of CD69 is represented as mean MFI ± SEM (**lower panels**). **A–C:** Significant differences were designated by using one-way analysis of variance with a Bonferroni posttest. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (n = 3 to 8, at least two independent experiments).

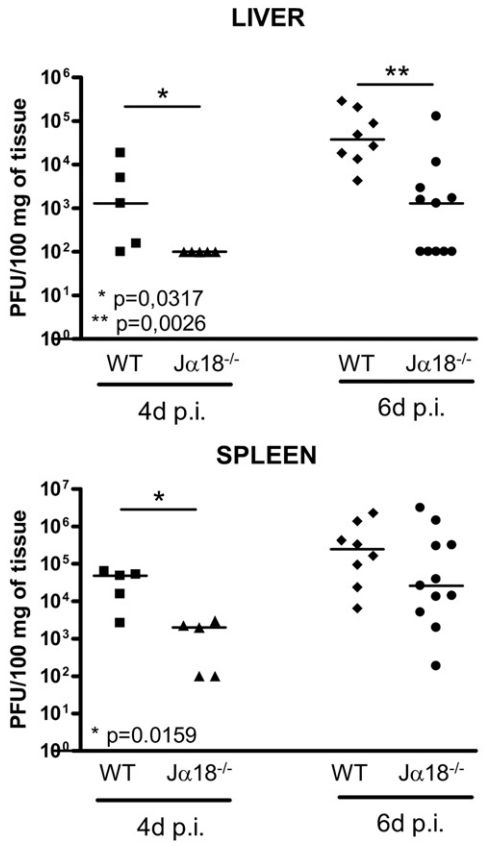

Figure 6. Viral load in WT and *Jα18*$^{-/-}$ mice infected with a lethal dose of DENV. Viral loads, as analyzed from infectious virus recovered from liver and spleen of WT and *Jα18*$^{-/-}$ mice, were assessed at 4 and 6 days after infection (p.i.) using 30 LD_{50}. Data are given as the log of PFUs/100 mg tissue. Results are expressed as the geometric means and are representative of at least two experiments (n = 5 to 11).

lower levels of CD62-L, a sign of activation, on DENV infection (Figure 5B). Neutrophils failed to become activated in infected *Jα18*$^{-/-}$ mice, at least insofar as CD62-L down-regulation. It has been suggested that CD8$^+$ and CD4$^+$ T-lymphocyte activation is associated with severity of dengue infection.[58] We observed a small decrease in the percentage of CD4$^+$ T cells but no changes in the percentage of CD8$^+$ T cells and in the spleen of DENV infected WT and *Jα18*$^{-/-}$ mice (Figure 5C). However, although both CD4$^+$ and CD8$^+$ T cells expressed more CD69 in DENV-infected WT mice, this enhancement was less important in infected *Jα18*$^{-/-}$ mice. Together, these results demonstrate less activation of immune cells in the absence of iNKT cells on DENV infection, revealing an important and new interplay between those cell types in this experimental model.

Infected Jα18$^{-/-}$ *Mice Have a Lower Viral Titer Than WT Animals*

Inasmuch as disease was less severe in *Jα18*$^{-/-}$ mice and iNKT cells may be important to deal with viral replication, it was important to evaluate the effects of iNKT cell deficiency on viral load. Viral particles were detected in the liver and spleen of both WT and *Jα18*$^{-/-}$ mice at 4 days after infection, and in particular at 6 days after infection (Figure 6). Viral load was significantly lower (except in spleen at 6 days after infection) in *Jα18*$^{-/-}$ mice than in their WT controls. Thus, in our experimental system, iNKT cell deficiency did not result in impaired containment and clearance of DENV during infection; on the contrary, it led to a decreased viral load. This suggests that iNKT cells not only favor inflammatory injury but also DENV replication.

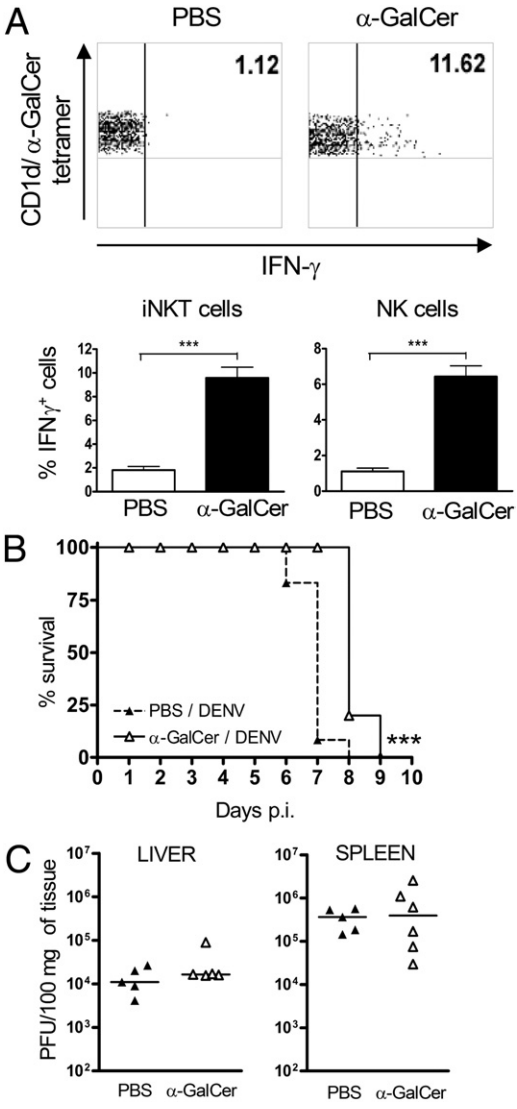

Figure 7. Treatment with α-GalCer failed to protect mice against DENV infection. **A:** Mice were injected i.v. with either 2 μg α-GalCer or vehicle alone. After 12 hours, splenic cells were labeled with CD1d/α-GalCer tetramer or TCRβ antibody, fixed, and permeabilized for intracellular cytokine staining. Cells were analyzed using flow cytometry, and gated CD1d/α-GalCer tetramer$^+$ TCRβ$^+$ (iNKT) cells were screened for intracellular IFN-γ production. Gates were set based on the isotype control. Mean ± SEM percentages of iNKT cells, as well as CD5$^-$ NK1.1$^+$ (NK) cells, positive for IFN-γ are represented (n = 6). ***$P < 0.001$. **B:** Percentages of survival (n = 10, two independent experiments). Mice were administered 2 μg α-GalCer i.v. at 1 hour before DENV (30 LD_{50}) challenge. Log-rank test for comparisons of Kaplan-Meier survival curves indicated a significant decrease in mortality of α-GalCer-inoculated mice compared with PBS-treated animals. ***$P <$ 0.001. **C:** Viral loads in liver and spleen of PBS- or α-GalCer–treated animals at 6 days after infection. One of two representative experiments is shown.

Exogenous Activation of iNKT Cells Has No Effect on DENV-Mediated Death and Viral Replication

Exogenous activation of iNKT cells by α-GalCer initiates anti-viral immunity that protects mice against viral challenge.[36,38,60–62] Because endogenous activation of iNKT cells failed to control DENV replication (Figure 6) and promoted disease in this system, we intended to investigate the consequences of α-GalCer therapy on DENV replication and DENV-associated death. At the dosage used, α-GalCer induced potent activation of iNKT cells (Figure 7A) and bystander activation of NK cells (Figure 7A) and dendritic cells (data not shown). Although α-GalCer inoculation before DENV challenge slightly delayed death in infected animals (Figure 7B), it did not affect the viral load (Figure 7C). Together, at least with the protocol used in this study, exogenous activation of iNKT cells with the superagonist α-GalCer failed to control DENV replication and virus-associated death.

Discussion

DENV causes the most prevalent arthropod-borne viral illness in humans worldwide. At present, the mechanisms of DENV-induced disease and immunity are poorly defined, and the protective versus pathogenic nature of the immune response to DENV infection is unclear. In particular, how the cytokine storm and shock, which characterize DENV disease, are induced and controlled remains elusive. Considering the role of iNKT cells in host defense mechanisms during infection and in the promotion and control of inflammatory responses in many systems, we questioned whether these cells have a role in the host response during experimental DENV infection. We used a mouse model of acute DENV infection that resembles the severe dengue infection that affects humans,[44–47] and our findings suggest a detrimental role for iNKT cells in severe DENV-associated disease and death.

To date, no studies have addressed the role of iNKT cells during infection with flaviviruses including the four serotypes of DENV, West Nile virus, yellow fever virus, and Japanese encephalitis virus. Their potential role during hepatitis C virus, a hepacivirus of the *Flaviviridae* family, has been addressed in the human system, although conflicting data about the frequency and function of iNKT cells in both liver and blood have been reported.[63–67] Viral infections can lead to activation of iNKT cells, and this may strongly affect control of the local immune response.[24–26] We first assessed the activation status of iNKT cells and their potential recruitment to and expansion in the spleen and liver during DENV infection. With use of a lethal dose of DENV serotype 2, our data show that no massive expansion of iNKT cells occurs in splenic and hepatic tissues during the early phase of infection. On the contrary, the apparent number of iNKT cells significantly decreased at 5 days after infection, a phenomenon probably due to TCR internalization after activation of these cells[49–51] or to activation-induced cell death.[52,53] At day 5 after infection, expression of the early activation marker CD69, as well as Fas-L, was increased on iNKT cells. Furthermore, iNKT cells express IFN-γ intracellularly at this time point. Of note, iNKT cells from mice inoculated with 0.3 LD_{50} DENV, a dose that causes minor loss of weight and death, failed to become activated insofar as CD69, Fas-L, and IFN-γ expression (data not shown). In aggregate, iNKT cells become activated at 5 days after severe DENV infection, a time point that just precedes the peak of viral replication at day 6. In contrast, iNKT cells remained unactivated in conditions in which only minor clinical signs were observed. The mechanisms (role of the CD1d molecule and inflammatory cytokines) by which iNKT cells become activated during severe DENV infection are currently being studied.

Excessive systemic inflammation is detrimental after DENV challenge, and infection of mice with DENV causes an acute inflammatory response, hemorrhagic manifestations, and thrombocytopenia, which eventually lead to death. In our experimental system, the potent local (liver) injury, vascular leakage, systemic inflammation, and uncontrolled virus replication were important determinants of the fatal outcome. Physiologically, iNKT cells can augment or inhibit inflammatory responses through a variety of mechanisms, depending on the context (ie, sterile or nonsterile inflammation) and the targeted organ. Several experimental models have highlighted the beneficial role of iNKT cells in virus-associated inflammatory responses.[27–32] However, they can also contribute to the immunopathogenesis of viral diseases. For example, using an experimental mouse model of chronic lung disease triggered by infection with Sendai virus, Kim et al.[33] demonstrated that IL-13 production by iNKT cells contributes to pulmonary disease. More recently, Stout-Delgado et al.[34] demonstrated that IL-17 production by iNKT cells from aged mice infected with herpes simplex virus 2 is sufficient to promote liver damage and death. In the context of sterile inflammation, iNKT cells can have deleterious effects on local and systemic responses. For example, iNKT cells strongly participate in hepatitis induced by concanavalin A[54,68] and α-GalCer,[69,70] in hepatic reperfusion injury,[71,72] and in lipopolysaccharide-induced[73] or cecal ligation and puncture-mediated[74] acute septic shock. We evaluated their potential regulatory function in DENV-induced disease. In multiple repeated experiments, it was consistently observed that, compared with WT animals, most mice lacking iNKT cells were resistant to severe DENV infection, and the other 30% died at later time points (between days 8 and 14). Of note, the adoptive transfer of NKT ($CD5^+$ $NK1.1^+$) cells into $J\alpha18^{-/-}$ mice failed to significantly restore the phenotype to that of WT animals, an effect that could be due to an interfering effect of vNKT cells. In some pathologic conditions, vNKT cells and iNKT cells exert opposing functions in immune regulation.[72,75–79] To verify this, $J\alpha18^{-/-}$ mice were reconstituted using pure iNKT cells. In this condition, although the kinetics of animal death was different than that of WT animals, 80% of mice died of infection. Together, iNKT cells have a key role in DENV-associated death. Lack of iNKT cells greatly ameliorated DENV-associated disease including vascular leakage syndrome, a hallmark of se-

vere DENV infection in humans.[6,8] Increased hematocrit, a marker of hemoconcentration, and Evans blue leakage were strongly reduced in DENV-infected $J\alpha18^{-/-}$ mice compared with infected WT mice. In parallel, histologic examination of liver sections revealed that, compared with WT mice, infected $J\alpha18^{-/-}$ developed less acute disease. Thus, in agreement with other studies that demonstrated a detrimental role of iNKT cells in liver disease,[54,68–72] our data strongly suggest that iNKT cells contribute to hepatic injury during DENV infection. Moreover, analysis of cytokine production indicated that iNKT cells orchestrate, either directly or indirectly, the high local and systemic inflammatory responses. The exact mechanisms by which iNKT cells contribute to DENV pathogenesis are yet to be defined. It is possible that they act through synthesis of inflammatory cytokines that are able to directly or indirectly promote injury. As described in concanavalin A–induced hepatic injury,[54] it is also possible that, through Fas or Fas-L interaction, iNKT cells mediate cytotoxic effects on hepatocytes, thus contributing to liver disease. In this system, the Fas/Fas-L pathway may also participate in vascular leakage by promoting apoptosis of endothelial cells.[80] Attempts are under way to investigate these issues and to identify other functions of iNKT cells in DENV-associated disease. In parallel, because iNKT cells and vNKT cells might have opposing roles during infection and inflammation,[72,75–79] it would be interesting to examine the role of both subsets and their interplay during DENV infection.

In contrast to experimental models involving other viruses,[27–31] iNKT cell deficiency did not lead to impaired containment and clearance of DENV in the liver and spleen in our experimental system. This finding is not without precedent because iNKT cells have been shown to be dispensable to control the virus load in some experimental systems.[32,36,81] The viral load was even diminished (by approximately fivefold to 50-fold) in $J\alpha18^{-/-}$ mice compared with WT animals. Previous findings have suggested that production of inflammatory mediators favors DENV replication *in vivo* and *in vitro*.[82–84] Thus, it is likely that in this infectious system, iNKT cells indirectly favor virus replication by promoting inflammation. Because the inflammatory response is strongly reduced in $J\alpha18^{-/-}$ mice, this positive feedback for viral replication is also down-regulated. Our data demonstrate that during DENV infection, iNKT cells produce IFN-γ and that iNKT cells *trans*-activate NK cells to produce it. Considering the critical function of IFN-γ in restricting DENV replication,[48,85] the lack of function of the iNKT cell/NK cell pathway in virus containment in this system is somewhat surprising. The role of NK cells during DENV infection is unclear. It has been proposed that they could exert cytotoxic functions on infected cells, although this activity has not yet been firmly proved *in vivo*.[86–88] Prophylactic administration of the iNKT cell superagonist α-GalCer is protective against a wide variety of viruses in rodent models,[36,38,60–62] irrespective of physiologic involvement of iNKT cells in resistance. Of note, at least with the protocol used in the present study (single injection 1 hour before challenge), prophylactic inoculation of α-GalCer failed to affect virus replication and was without effect on mouse survival, although a slight delay in death was observed relative to vehicle-injected animals. This is the first time, to our knowledge, that α-GalCer has been described as ineffective during virus infection. This finding confirms the lack of iNKT cell positive functions on DENV replication after natural activation and infers that IFN-γ production by iNKT cells and NK cells has a minor, if any, effect in this setting.

In conclusion, our data demonstrate a key role for iNKT cells in development of disease and in death associated with an acute model of experimental DENV infection. Although less frequent, human iNKT cells share many functional characteristics with mouse iNKT cells. In view of the data described herein, and based on a mouse model, it would be interesting to investigate the potential role of iNKT cells during acute DENV infection in the human system. For this, analysis of the activation status of iNKT cells in patients infected with DENV will be of interest. A better understanding of the mode of *in vivo* iNKT cell activation and of their precise functions in DENV infection may enable development of novel therapeutic approaches such as iNKT cell antagonists.

Acknowledgments

We thank the National Institute of Allergy and Infectious Diseases Tetramer Facility at Emory University for supplying CD1d tetramers and Drs. Toshinori Nakayama and Masaru Taniguchi for the gift of $J\alpha18^{-/-}$ C57BL/6 mice. We also thank Louis Fauconnier and Catherine Vendeville for technical assistance and Prof. Danielle G. Souza (UFMG, Brazil) for her kind material support.

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